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<b>(21) International Application Number:</b> PCT/US91/05179 <b>(22) International Filing Date:</b> 29 July 1991 (29.07.91) <b>(30) Priority data:</b> 562,002                      2 August 1990 (02.08.90)                      US <b>(71) Applicant:</b> BIOCARB, INC. [US/US]; 300 Professional Drive, Gaithersburg, MD 20879 (US). <b>(72) Inventors:</b> KRIVAN, Howard, C. ; 7703 Ironforge Ct., Derwood, MD 20855 (US). SAMUEL, James, E. ; 15902 Indian Mills Terrace, Derwood, MD 20855 (US). <b>(74) Agent:</b> KARNY, Geoffrey, M.; Dickstein, Shapiro & Morin, 2101 L Street, N.W., 4th Floor, Washington, DC 20037 (US).		<b>(81) Designated States:</b> AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> ADHESION RECEPTORS FOR PATHOGENIC OR OPPORTUNISTIC MICROORGANISMS  <b>(57) Abstract</b>  Disclosed herein are receptors for pathogenic or opportunistic microorganisms, methods of obtaining such receptors, and methods of using such receptors for diagnostic or pharmaceutical purposes. The receptor comprises a substantially pure compound selected from the group consisting of GalB1-4GlcNAcB1-3GalB1-4GlcB1-1-X(R), GalB1-3GlcNAcB1-3GalB1-4GlcB1-1-X(R), GlcNAcB1-3GalB1-4GlcB1-1-X(R), GalB1-4GlcNAcB1-3GalB1-4Glc, GalB1-3GlcNAcB1-3GalB1-4Glc, GlcNAcB1-3GalB1-4Glc, GalB1-4GlcNAcB1-3Gal, and GalB1-3-GlcNAcB1-3Gal wherein X is sphingosine, hydroxylated sphingosine, or saturated sphingosine and R is H or an N-acyl fatty acid derivative of X such that X(R) is a ceramide. The invention further comprises proteins and polypeptides that bind to the receptors, methods of obtaining such proteins or polypeptides from natural sources or through recombinant DNA techniques, and methods of using the purified proteins and polypeptides for pharmaceutical and diagnostic purposes, preferably in a vaccine for administration to an animal or human host to protect against pathogenic or opportunistic microorganisms.		

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**ADHESION RECEPTORS FOR PATHOGENIC  
OR OPPORTUNISTIC MICROORGANISMS**

**FIELD OF THE INVENTION**

This invention relates to certain receptors for pathogenic and opportunistic microorganisms. The receptors may be used in compositions, kits, apparatus, and methods for detecting or measuring pathogenic or opportunistic microorganisms, removing such microorganisms from a liquid, and treating infection or other illness caused by such microorganisms.

The invention further relates to an isolated microbial adhesin protein that binds to the receptors and to methods of obtaining such protein. Such methods include the production of the recombinant protein by genetic engineering means. The protein is expected to be useful in diagnostic and therapeutic compositions and particularly in a vaccine for the protection of a human or animal host from infection or other disease caused by pathogenic or opportunistic microorganisms.

**REFERENCES**

Several publications are referenced herein by Arabic numerals within parenthesis. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are hereby incorporated herein by reference in their entirety, unless otherwise noted.

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BACKGROUND OF THE INVENTION

The process by which microorganisms bind to host cells is called adherence or adhesion, and it is now generally well accepted that this mechanism is an important step in the initiation of microbial colonization and infection. It has been known for a long time that specific adhesion of bacteria, fungi, mycoplasmas, and viruses to host cells is mediated by cell surface carbohydrate structures (1,2). This is not surprising because all animal and human cells are sugar-coated. That is, they are all covered with a layer of carbohydrate that occurs in the form of glycoproteins and glycolipids which are anchored in the cell membrane and are the glycomolecules that many microorganisms first come into contact with during the infection process. Generally, the structures on the microorganism that mediate binding of infectious agents to host cells are called adhesins, and the host cell carbohydrate structures recognized by microbial adhesins are called the receptors. Therefore, the presence of receptors on host tissue is just as much as a determinant of microbial infectivity as is the microorganisms' display of adhesins.

Cell surface carbohydrates have been implicated as receptors for infection based mainly by indirect studies, such as the inhibition of microbial adhesion or hemagglutination by simple sugars and/or by the pretreatment of cells with carbohydrate-splitting enzymes called glycosidases (2,3). Only recently has a more systematic study been conducted using direct binding assays. One such assay involves overlaying glycolipid chromatograms with labeled microorganisms, and, in this

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way, a number of glycolipids may be shown to bind to a particular bacterium or virus. Because glycolipids contain one oligosaccharide per molecule (unlike glycoproteins which contain several different saccharides per molecule), the specific carbohydrate receptor sequence is much more easily determined (4). Using the overlay technique, it was demonstrated that the E. coli that cause urinary tract infections in humans bind to Gal-alpha-1-4Gal-containing glycolipids (5). This result was the impetus for a more thorough investigation of glycolipids as receptors for infectious agents and toxins.

To date, glycolipid receptors have been described for a variety of microorganisms and toxins. For example, species of Propionibacterium bind to lactosylceramide (GalB1-4GlcB1-1Cer) (6,7), many pulmonary pathogenic microorganisms, including Pseudomonas aeruginosa, Haemophilus influenzae, and Streptococcus pneumoniae, bind to ganglio-series glycolipids containing GalNacB1-4GalB1-4Glc sequences (8), and Mycoplasma pneumoniae binds to sulfated glycolipids containing Gal(3So<sub>4</sub>)B1-1Cer sequences (9). (As used in the formulas in this application, B equals beta.) In addition, it has been reported that Streptococcus pneumoniae binds to glycolipids containing the disaccharide GlcNacB1-3Gal (10, 11). Recently, Neisseria gonorrhoeae has also been reported to bind to ganglio-series glycolipids containing GalNacB1-4GalB1-4Glc sequences (12,13).

The inventors have discovered still another receptor that, surprisingly, appears to be a universal receptor for pathogenic and opportunistic microorganisms. With such a receptor, a purified universal adhesin protein,

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the gene or genes coding for it, and recombinant proteins produced by such genes are obtained. The protein will be useful in a broad spectrum vaccine against these microorganisms as well as in diagnostic and therapeutic compositions.

#### SUMMARY OF THE INVENTION

It is an object of the invention to provide receptors for pathogenic or opportunistic microorganisms.

It is another object of the invention to provide compositions useful as receptors for pathogenic or opportunistic microorganisms.

A further object of the invention is to provide diagnostic kits and methods for detecting the presence of pathogenic or opportunistic microorganisms in a sample.

Yet another object of the invention is to provide a method and apparatus for removing pathogenic or opportunistic microorganisms from a liquid.

Still another object of the invention is to provide pharmaceutical methods and compositions for the treatment, prevention, or amelioration of illness or infection in animals caused by pathogenic or opportunistic microorganisms.

A further object of the invention is to provide an isolated microbial adhesin protein useful in diagnostics, therapeutics, and vaccines.

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A still further object of the invention is to provide DNA coding for the microbial adhesin protein, vectors containing the DNA, and microorganisms transformed by such DNA and vectors.

Additional objects and advantages of the invention will be set forth in part in the description that follows, and in part will be obvious from the description, or may be learned by the practice of the invention. The objects and advantages of the invention will be attained by means of the instrumentalistics and combinations particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purpose of the invention, as embodied and broadly described herein, the present invention provides a receptor for a pathogenic or opportunistic microorganism comprising a substantially pure compound selected from the group consisting of GalB1-4GlcNACB1-3GalB1-4GlcB1-1-X(R), GalB1-3GlcNACB1-3GalB1-4GlcB1-1-X(R), GlcNACB1-3GalB1-4GlcB1-1-X(R), GalB1-4GlcNACB1-3GalB1-4Glc, GalB1-3GlcNACB1-3GalB1-4Glc, GlcNACB1-3GalB1-4Glc, GalB1-4GlcNACB1-3Gal, and GalB1-3-GlcNACB1-3Gal where X is sphingosine, hydroxylated sphingosine, or saturated sphingosine and R is H or an N-acyl fatty acid derivative of X such that X(R) is a ceramide. Preferably, such receptor is a pure compound from the preceding group attached to a soluble or insoluble substrate. Most preferably, the receptor is attached to a liposome.

In another embodiment, the invention comprises a method for detecting the presence of pathogenic or opportunistic microorganisms in a sample suspecting of

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containing such microorganisms. The sample is contacted with receptors of the invention for a period of time and under conditions sufficient for the receptors to bind to the microorganisms if they are present in the sample. A determination is then made whether or not the receptors have bound to the microorganisms. Preferably, the receptors are attached to an insoluble substrate and the complexes are detected by a detectably labeled antibody to an antigen on the surface of the microorganism.

The invention further comprises a diagnostic kit for the detection of these microorganisms in a sample. The kit contains the receptors of the invention attached to an insoluble support and means for detecting or measuring the formation of complexes of the receptors and the microorganisms or the receptors and the adhesin proteins from the microorganisms. The kit is particularly useful for detecting pathogenic or opportunistic microorganisms in a sample that is a body fluid from a human or other animal. Preferably, the means for detecting or measuring the complexes is an immunoassay.

In another aspect, the invention is a method and apparatus for removing these microorganisms from a liquid. The liquid is contacted with the receptors of the invention for a period of time and under conditions sufficient to bind the receptors to the microorganisms. The liquid is then removed from contact with the receptors, thereby removing the microorganisms from the liquid. The related apparatus comprises a container for holding the liquid, means for introducing the liquid into a container, and means for removing it from the container. The container



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contains the receptors of the invention, preferably bound to a solid support within the container.

The invention further comprises methods and pharmaceutical compositions for the treatment, prevention, or amelioration of infection or other diseases in humans and other mammals caused by pathogenic or opportunistic microorganisms. An effective amount of the receptors of the invention for such treatment, prevention, or amelioration is administered to the mammalian host, preferably in combination with a pharmaceutically acceptable carrier. Preferably, the carrier is a liposome. In an alternative preferred embodiment, the carrier is a macromolecule to which the receptor has been coupled. In a particularly preferred embodiment, the receptor is dissolved or suspended in a liquid that is physiologically compatible with mammalian cells and such composition is used to inhibit the adherence of pathogenic or opportunistic microorganisms to such cells or to remove such microorganisms from such cells.

In still another embodiment, the invention provides a substantially pure microbial adhesin protein, obtained from the surface of a pathogenic or opportunistic microorganism, that binds to the receptors of the invention. The protein is obtained by solubilizing the membranes of pathogenic or opportunistic microorganisms. The solubilized material contains the adhesin protein. This material is separated from the insoluble material and contacted with the receptors of the invention for a period of time sufficient for the protein molecules to bind to the receptors. The receptors are attached to an insoluble solid support. The protein molecules are then removed from

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the receptors and recovered. Preferably, the receptors are washed after being contacted with the solubilized material to remove unbound material completely.

The invention also includes modified proteins and polypeptides derived from the adhesion protein, provided such derived proteins and polypeptides bind to the receptors of the invention. Preferably, such derivatives are one or more of the epitopes of the adhesin protein. Preferably, they are also immunogenic for human or animal hosts and immunologically cross reactive with the bacterial adhesin protein. In a particular preferred embodiment, the polypeptide has been altered to enhance its immunogenicity.

The polypeptides are useful in a vaccine for preventing, ameliorating, or treating infection or other disease in a human or animal host. In a preferred embodiment, the vaccine comprises an immunologically effective amount of a protein or polypeptide of the invention in a pharmaceutically acceptable carrier. In an alternative preferred embodiment, the vaccine comprises an avirulent microorganism which has been genetically engineered to express a protein or polypeptide of the invention.

The proteins and polypeptides of the invention are preferably recombinant proteins and polypeptides that have been produced through genetic engineering techniques. They are produced by an appropriate host cell that has been transformed by DNA that codes for such proteins or polypeptides.

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An isolated or substantially pure DNA sequence that codes for the microbial adhesin protein of the invention is obtained as follows. Receptors of the invention are used to screen a genomic library containing the DNA of a pathogenic or opportunistic microorganism. The library is made of clones of vectors into which different sequences of the DNA have been operably and recoverably inserted, with each of the vectors containing only one sequence of the DNA. The clones are contacted with the receptors of the invention to identify a clone that binds to the receptors. The clone is then isolated. Preferably, the exogenous DNA sequences are recovered from the clone.

The invention further comprises isolated or substantially purified DNA derived from this DNA, for example, by single or multiple mutations. Preferably, such DNA hybridizes with the DNA obtained from the genomic library under conditions of moderate stringency.

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one embodiment of the invention and, together with the description, serve to explain the principles of the invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the binding of  $^{125}\text{I}$ -labeled N. gonorrhoeae strain MS11<sub>mk</sub> (P-, PII-) to paragloboside and lacto-N-triaosylceramide separated by thin-layer chromatography. Glycolipids were chromatographed on aluminum-backed silica gel HPTLC plates developed in chloroform/methanol/0.25% KCl in water, 5:4:1. The plates

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were coated with 0.1% polisobutylmethacrylate, soaked in TBS-BSA, and incubated for 2 h at 25° C with  $^{125}\text{I}$ -labeled gonococci suspended in HBSS-BSA (panel B), or sprayed with orcinol reagent to identify glycolipids (Panel A). Lanes 1, 1 ug each galactosylceramide (CMH), lactosylceramide doublet (CDH), trihexosylceramide (CTH), globoside (GL4), Forssman glycolipid (FORS), and gangliosides GM3, GM2, GM1, GD3, GD1a, GD1b, and GT1b; lanes 2, 1 ug sialylparagloboside; lanes 3, 1 ug paragloboside derived from sialylparagloboside by treatment with neuraminidase; lanes 4, lacto-N-triaosylceramide derived from paragloboside by treatment with beta-galactosidase; lanes 5, 1 ug lactosylceramide derived from lacto-N-triaosylceramide by treatment with N-Acetyl-B-hexosaminidase. For structures see Table I.

Figure 2 shows the binding of N. gonorrhoeae strain MS11<sub>mk</sub> (P-, PII-) to immobilized glycolipids. Lipids in 25 ul of methanol containing 0.1 ug each of the auxiliary lipids cholesterol and phosphatidylcholine were evaporated in flat-bottom wells of polyvinylchlorine microtiter plates. The wells were blocked with 1% albumin overnight at 4° C, washed twice with HBSS-BSA and incubated at 25° C with 25 ul of  $^{125}\text{I}$ -labeled N. gonorrhoeae (approximately  $10^5$ cpm). After 2h, the wells were washed 5 times with saline, cut from the plate, and bound radioactivity quantified in a scintillation counter. In control experiments gonococci were incubated with auxiliary lipids only to correct for nonspecific binding (typically < 1% of the total radioactivity added). N. gonorrhoeae binding was determined in HBSS-BSA for asialo-GM2 (■), paragloboside (▲) and lactosylceramide, GM1 or sialylparagloboside (□).

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Figure 3 shows the binding of anti-LOS monoclonal antibody 4BE12 to authentic paragloboside. Panel A, glycolipids detected by orcinol staining. Panel B, autoradiogram of chromatogram overlay with monoclonal antibody B54 and radiolabeled anti-mouse IgM antibody. Lanes 1, 1 ug each of galactosylceramide (CMH), lactosylceramide (CDH), trihexosylceramide (CTH), globoside (GL4), Forssman glycolipid (FORS), and gangliosides GM3, GM2, GM1, GD3, GD1a, GD1b, and GT1b; lanes 2, 1 ug asialo-GM1; lanes 3, 1 ug of paragloboside; lanes 4,5,6,7,8, and 9 are 0.5, 0.25, 0.125, 0.062, 0.031, and 0.016 ug of paragloboside, respectively.

Figure 4 shows the binding of Pseudomonas aeruginosa CT4 to glycolipids separated by thin layer chromatography. Glycolipids were chromatographed on aluminum-backed silica gel HPTLC plates developed in chloroform/methanol/0.25% KCl in water, 5:4:1. The plates were coated with polyisobutylmethacrylate, soaked in Tris-BSA, and incubated for 2 h at 25°C with <sup>125</sup>I-labeled P. aeruginosa suspended in Tris-BSA containing 1% BSA. Figure 4A shows standard glycolipids detected with orcinol reagent. Figure 4B shows glycolipid receptors detected by overlaying with radiolabeled microorganisms followed by autoradiography (18 h). Lanes 1, galactosylceramide (CMH), lactosylceramide (CDH), sulfatide (SFT), globotriaosylceramide (CTH), globoside (GL4), and gangliosides GM3, GM2, GM1, GD1a, GD1b, and GT1b (1 ug of each glycolipid); lanes 2, gangliotriaosylceramide (asialo-GM2, 1 ug) and gangliotetraosylceramide (asialo-GM1, 1 ug); and lanes 3, lacto-N-tetraosylceramide doublet (paragloboside, 2 ug).

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DETAILED DESCRIPTION OF THE INVENTION

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the following examples, serve to explain principles of the invention.

The invention relates to a universal adhesion receptor for pathogenic or opportunistic microorganisms, microbial adhesin proteins and polypeptides that bind to the receptor, DNA coding for the proteins, and methods of using these materials. As used herein, the terms "adhesin receptor" or "receptor" mean a compound that has a specific affinity for and selectively binds to one or more types of microorganism.

The receptor comprises certain lacto-series glycolipids and derivatives thereof that act as adhesion receptors to a broad and diverse spectrum of microorganisms. One of the glycolipids is lactotriaosylcer (lactotriaosylceramide, N-acetylglucosamine-beta 1-3-galactose-beta 1-4-glucose-beta 1-1-ceramide), which is represented by the following formula:

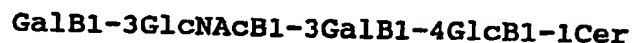


Another is paragloboside (lactoneotetraosylceramide, galactose-beta 1-4-N-acetylglucosamine-beta-1-3-galactose-beta 1-4-glucose-beta-1-1-ceramide), which is represented by the following formula:

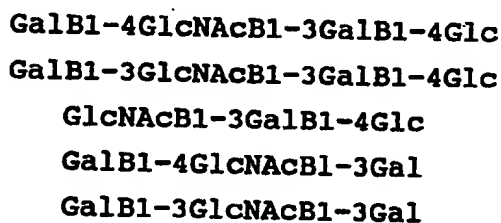


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A third is an isomer of paragloboside, which is represented by the following formula:



(Trivial names and structures are represented according to the recommendations in Reference 38 and the references cited therein. As used in the formulas in this application, B equals beta.) In addition, the receptors of the invention include the above-referenced compounds from which the fatty acid moiety of the ceramide moiety has been removed. Finally, the receptors of the invention include the carbohydrate moieties of the above-referenced structures and certain residues thereof. These are represented by the following formulas:



The preferred receptor structure is GlcNAcB1-3GalB1-4GlcB1-1Cer.

Therefore, the receptors of the invention comprise a substantially pure compound selected from the group consisting of GalB1-4GlcNAcB1-3GalB1-4GlcB1-1-X(R), GalB1-3GlcNAcB1-3GalB1-4GlcB1-1-X(R), GlcNAcB1-3GalB1-4GlcB1-1-X(R), GalB1-4GlcNAcB1-3GalB1-4Glc, GalB1-3GlcNAcB1-3GalB1-4Glc, GlcNAcB1-3GalB1-4Glc, GalB1-4GlcNAcB1-3Gal, and GalB1-3-GlcNAcB1-3Gal wherein X is sphingosine,

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hydroxylated sphingosine, or saturated sphingosine and R is H or an N-acyl fatty acid derivative of X such that X(R) is a ceramide.

The receptors of the invention are at least substantially pure and preferably pure. As used herein, the term "substantially pure" and similar terms means that the compound is at least 80% pure by weight. That is, the compound does not contain more than 20% by weight of chemicals other than the compound. As used herein, the term "pure" and related terms means that the receptor compound provides only one band after being analyzed by thin layer chromatography and chemically stained according to the methods described in Reference 9, incorporated herein by reference. If necessary, the compound can be further purified by known techniques. Thus, the receptor compound is at least 90% pure by weight, preferably at least 95% pure by weight, and most preferably at least 98% pure by weight.

As mentioned previously, the receptors of the invention act as adhesion receptors for a broad array of pathogenic or opportunistic microorganisms. As used herein, the term "microorganisms" include bacteria, viruses, mycoplasma, fungi, rickettsiae, and protozoa. Preferably, the microorganisms are bacteria. As used herein, the term "pathogenic microorganisms" means any microorganisms that cause disease (including infection) or morbid symptoms in humans or other animal hosts. As used herein, the term "opportunistic microorganisms" means any microorganisms that do not ordinarily cause disease or infection but which become pathogenic under certain circumstances in which the host organism is stressed, such



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as having an impaired immune response. Preferably, the receptors of the invention bind to bacteria of the following genera: Streptococcus, Staphylococcus, Clostridium, Borrelia, Haemophilus, Pseudomonas, Neisseria, Coxiella, and Shigella. Most preferably, the bacteria are the following: Streptococcus pneumoniae, Streptococcus agalactiae (Gp. B), Staphylococcus aureus, Clostridium perfringens, Clostridium difficile, Borrelia burgdorferi (Lyme agent), Haemophilus influenzae, Haemophilus parainfluenzae, Pseudomonas aeruginosa, Pseudomonas cepacia, Pseudomonas maltophilia, Neisseria gonorrhoeae, Neisseria meningitidis, Shigella dysenteriae, Shigella flexneri, and Coxiella burnetti. In an alternative preferred embodiment, the microorganisms are rotaviruses.

The inventors have also surprisingly discovered that the disaccharide, GlcNAcB1-3Gal, which has been reported as a receptor for S. pneumoniae but not for any of the other microorganisms mentioned above, also acts as a receptor for the other above-referenced microorganisms. Thus, the invention also includes compositions containing this receptor and methods of using it with respect to microorganisms other than S. pneumoniae.

The receptors of the invention are prepared by known techniques in conjunction with the teachings disclosed herein. Paragloboside is prepared by desialylation of erythrocyte sialylparagloboside with 1 M formic acid for 60 minutes at 100°C as disclosed in Reference 9. Lactotriaosylcer is prepared by digestion of paragloboside with beta-galactosidase as described for the preparation of asialo-GM2 from asialo-GM1 in Reference 14. The lyso derivatives of these compounds are prepared by

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techniques disclosed in Basta, M., Karmali, M., and Lingwood, C. (1989) J. Chm. Microbiol. 27: 1617-1622, incorporated herein by reference. The carbohydrate moiety derivatives of these glycolipids are prepared by removing the ceramide moiety according to the techniques of Miljkovic, M. and Schengrund (1986) Carbohydr. Res. 155: 175-181; Ito, M. and Yamagata, T. (1986) J. Biol. Chem. 261: 14278-14282; and Kanfer, J.N. and Hakomori, S-i (1983) Handbook of Lipid Research, Sphingolipid Biochem., Plenum Press, NY, incorporated herein by reference, or synthesized directly according to the techniques of European patent application 84850084.9 of Svenska Sockerfabriks Ab, published November 21, 1984 (Publication No. 0,126,043), incorporated herein by reference. The receptor GlcNAcB1-3GalB1-4Glc is synthesized according to the latter techniques.

The receptors of the invention are useful for detecting the presence of pathogenic or opportunistic microorganisms in a sample suspected of containing such microorganisms. In addition, since adhesins may be secreted by some microorganisms, the presence of the adhesins may be detected with the receptor. The receptors of the invention are contacted with the sample for a period of time and under conditions sufficient for receptors to bind to the microorganisms and/or the adhesins, if either or both are present. Such time and conditions can be readily determined by persons skilled in the art with respect to any particular type of microorganisms, given the teachings described herein. One then determines if the receptors have bound to the microorganisms and/or adhesins, forming microorganism-receptor and/or adhesin-receptor complexes. The binding of the microorganisms and/or the

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adhesins to the receptors is determined by techniques known to those skilled in the art in conjunction with the teachings disclosed herein.

Preferably, the receptors are bound to a solid matrix or other insoluble substrate. The receptors should be bound to the substrate in an amount and manner that allows sufficient binding of the microorganisms to be detected. Preferably, the receptors are bound as a molecular monolayer that substantially covers the surface of the substrate. The actual concentration of the receptor to a given substrate will depend upon the particular microorganism to be detected, the particular receptor being used, the particular substrate, and the binding efficiency of the receptor to the microorganism.

The receptor may be bound to the substrate in any suitable manner. Covalent, noncovalent, or ionic binding may be used. For example, the lipid portion may be hydrophobically bonded to certain plastic substrates. Covalent bonding can be accomplished by attaching the receptor to reactive groups on the substrate directly or through a linker arm as disclosed in U.S. Patent No. 4,657,849 to Kallenius et al., incorporated herein by reference. Alternatively, the lyso derivatives of the receptors can be attached to the solid support in accordance with the method disclosed in published European Patent Application No. 89113785.3 (publication no. 0 352 766), filed July 26, 1989 and published January 31, 1990, incorporated herein by reference.

Preferably, the receptor is first coupled to a soluble substrate, such as a protein. Bovine serum albumin

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is preferred. Then this combination is attached to the insoluble support.

The insoluble substrate may be any solid, insoluble material to which the receptors can be bound and which may be conveniently used in the assay of the invention. Such substrates include permeable and semipermeable membranes, glass beads, plastic beads, latex beads, plastic microtiter wells, agarose, dextran, sepharose and diatomaceous earth. Alternatively, the receptors may be bound to any porous or liquid permeable material, such as a screen or net. A binder may be used as long as it does not interfere with the ability of the receptor to bind microorganisms.

In a particularly preferred embodiment, the receptors are incorporated into liposomes. The liposomes are preferably prepared according to the techniques of Gruner, et al., *Biochemistry*, 24:2833-2842 (1985), incorporated herein by reference. The receptors are incorporated into the liposomes using standard techniques. They are essentially incorporated spontaneously into the lipid bilayer of the liposome after being contacted with the liposome. The fatty acid part goes into the lipid membrane, and the carbohydrate moiety sticks out of the membrane, interior or exterior to the liposome.

After the sample has been contacted with the substrate containing the receptors for a sufficient period of time to allow the microorganisms to bind to the receptors, such binding is detected through the application of the appropriate detecting means. Basically, the substrate containing the receptors and suspected of containing microorganisms bound to the receptors is

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contacted with a material that binds specifically to the microorganisms sought to be detected. Such materials include, for example, antibodies against the specific microorganisms, particularly against surface proteins, or a receptor that binds specifically to the microorganisms sought to be detected. Generally, the substrate is washed to remove all or substantially all unbound materials. The detecting assay may be an immunoassay, agglutination assay, thin layer chromatography assay, or cytotoxicity assay. Immunoassays include radioimmunoassays, enzyme-linked immunosorbent assays (ELISA), Western blot, immunofluorescent assays, chemiluminescent assays, and bioluminescent assays. The degree or amount of binding can also be determined through the application of known techniques, providing a measurement of the amount or concentration of microorganisms in the sample.

The antibody or carbohydrate receptor may be labelled with a substance that is easily detected. Such detectable moieties include an enzyme or radioactive, fluorescent, or chemiluminescent entity. A first antibody to a surface antigen of the microorganisms sought to be detected can be used in conjunction with a labelled second antibody to the first antibody. The first antibody is brought into contact with the substrate, binding to any microorganisms bound to receptors on the substrate. The substrate is preferably washed and contacted with the second antibody, which is then detected, thereby indicating the presence of the microorganisms on the substrate.

The various detectable moieties that can be used to label the antibodies or receptors used in such assays, the techniques for doing so, and the various specific assays in

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conditions for using them are well-known to those skilled in the art. For example, see U.S. Patent No. 4,486,530 to David et al., issued December 4, 1984, U.S. Patent No. 4,708,818 to Montagnier et al., issued November 24, 1987, and U.S. Patent No. 4,753,873 to Beltz et al., issued June 28, 1988, all of which are incorporated herein by reference. A particularly preferred assay incorporating the receptors of the invention is based upon the one disclosed in Basta, et al., J. Clin. Microbiology, 27:1617-1622 (1989), incorporated herein by reference.

Various types of samples can be tested for the presence of pathogenic or opportunistic microorganisms in accordance with the invention. The sample may be a biological sample, comprising or derived from a bodily fluid or tissue extract from a human or other animal patient. The sample will normally be diluted with an appropriate solution, such as physiological saline. Alternatively, a cotton swab or other material can be used to collect the microorganisms. The swab is then placed in a sterile solution in order to release the microorganisms into the solution. The solution is then tested in accordance with the invention.

The invention may also be used to test for the presence of microorganisms in environments that should be sterile, such as hospital operating rooms, drug and medical device manufacturing facilities, and food manufacturing facilities. Microorganisms can be collected by various means known to those skilled in the art, and a liquid sample containing such microorganisms can be prepared.

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The invention also comprises kits for detecting the presence of pathogenic or opportunistic microorganisms, which would have utility in scientific research, clinical diagnosis, and food safety procedures. The kit comprises: (1) a container holding a solid support or other insoluble substrate to which the receptors of the invention are attached; and (2) a means for detecting or measuring the formation of complexes of the microorganisms or adhesins and receptors. Alternatively, the receptors may be attached to the walls of the container that are exposed to the sample. Preferably, the insoluble substrate is the well of a microtiter plate. The detecting or measuring means is preferably a reagent containing a detectable moiety and capable of binding to the microorganisms. Such reagent may be the receptor of the invention conjugated with a detectable moiety. Alternatively, the reagent may be an antibody to a surface antigen of the microorganisms conjugated to a detectable moiety, such as an enzyme. In another alternative embodiment, the detecting or measuring means is a first antibody to a surface antigen of the bacterium and a second antibody to the first antibody wherein the second antibody is conjugated to a detectable moiety. An example of such a second antibody is goat anti-IgG labelled with peroxidase.

The invention also comprises methods and apparatus for removing pathogenic or opportunistic microorganisms from a liquid. The liquid is contacted with the instant receptors for a period of time and under conditions sufficient to bind the microorganisms to the receptors. Then the liquid is removed from contact with the receptors or the receptors are removed from contact with the liquid, thus providing a liquid which is free of the microorganisms

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or in which their number has been substantially reduced, depending upon the degree of removal sought. Preferably, the receptors are attached to a solid support as previously discussed herein and the liquid flows across the receptors.

The related apparatus comprises a container for the liquid with an inlet for introducing the liquid and an outlet for removing it. In addition, the container contains the receptors bound to a solid support or, alternatively, the receptors can be bound to the surface of the container exposed to the liquid. Most preferably, the container is a chromatographic column containing a solid phase matrix to which the receptors of the invention are attached. For example, they may be covalently coupled to epoxy-activated Sepharose (Sepharose-4B, Pharmacia) or a similar agarose-based matrix. The liquid is introduced to the top of the affinity column and the microorganisms are removed as the liquid passes down the column to be collected at the bottom.

In an alternative embodiment, the solid support for the receptors is a membrane, preferably a semipermeable membrane, or a liposome. As the liquid passes through the membrane or liposome, microorganisms are removed.

The receptors may also be used for treating, preventing, or ameliorating illness or infection in a host caused by pathogenic or opportunistic microorganisms. Generally, the receptors are administered in a pharmaceutically compatible carrier. Accordingly, the invention encompasses a pharmaceutical composition for the treatment, prevention, or amelioration of illness or infection in animals, particularly mammals, and most



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particularly humans, comprising an effective amount of the receptors of the invention for such treatment, prevention, or amelioration in a pharmaceutically acceptable carrier. The receptors may be in admixture with the carrier or coupled to it. For example, receptors may be coupled to a macromolecular carrier. They may also be within liposomes.

The compositions are prepared by techniques known in the art, given the teachings contained herein. The receptors are mixed with additives customary for pharmaceutical purposes, such as vehicles, stabilizers, solubilizers, or inert diluents, and converted by customary methods to a suitable administration form, such as tablets, capsules, solutions, suspensions, or emulsions. If the receptor is coupled to a macromolecular carrier, it is preferred that the carrier be a natural or synthetic polymer. Preferably, such polymer is a polypeptide, such as bovine serum albumin, or a polysaccharide. In a particularly preferred embodiment, the receptors are incorporated into liposomes as previously discussed herein.

The pharmaceutical preparations of the invention are administered locally, as by injection or topical application, intravenously, orally, intradermally, subcutaneously, intraocularly, subconjunctively, intramuscularly, and intrathecally. The mode of administration will necessarily depend upon the disease and microorganisms involved.

An effective amount of the composition is administered to the host or patient. The host or patient is an animal, preferably a mammal, and most preferably a human. The actual amount of the receptor to be

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administered will depend upon the particular disorder being treated. Such a determination is routinely made by those of ordinary skill in the art of determining therapeutic dosages and is within the scope of tasks routinely performed by them without undue experimentation.

A preferred use of the receptors of the invention is for inhibiting the adherence of pathogenic or opportunistic microorganisms to animal, preferably mammalian, cells or removing such microorganisms from such cells. The cells may be in tissue culture or in the host. A sufficient amount of the receptors of the invention to inhibit the adherence of such microorganisms or to remove such microorganisms are administered to such cells. Preferably, the receptors are dissolved or suspended in a liquid which is physiologically compatible with the cells.

In a particularly preferred embodiment, the receptors are dissolved in saline solution, which is then used to irrigate the wounded tissue of a mammalian host. For example, the tissue may be burned human skin tissue. Irrigation with the liquid helps remove microorganisms that could cause or are causing the wound to be infected.

In an alternative and preferred embodiment of the invention, the soluble substrate to which the receptors are coupled is a pharmaceutical compound directed against a type of pathogenic or opportunistic microorganisms. For example, the compound may be an antibiotic which is needed to treat disease caused by pathogenic bacteria, but which has serious side effects. By coupling the antibiotic to a receptor of the invention, the antibiotic will be targeted to the bacteria in question. This will allow the

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administration of a much lower, but still therapeutic dose to the host, lowering or eliminating the side effects attributable to the antibiotic. The compound may also be an immunogen that stimulates the production of antibodies against a particular microorganism in the host.

The invention further comprises an isolated microbial adhesin protein, obtained from the surface of a pathogenic or opportunistic bacterium, that binds to the receptors of the invention. The protein is obtained from natural sources, i.e., pathogenic or opportunistic microorganisms that bind to the receptors of the invention, by the application of standard protein purification techniques in view of the teachings contained herein. Alternatively, the protein is obtained as a recombinant protein through the application of standard genetic engineering techniques in view of the teachings contained herein.

The preferred method for producing the isolated protein from natural sources is as follows. Membranes of pathogenic or opportunistic microorganisms are obtained by standard techniques and solubilized, using a solubilizing compound, such as a detergent. The adhesin protein is in the solubilized material. The remaining insoluble material from the membrane is separated, preferably by centrifuging. The supernatant is passed through an affinity chromatography column containing the receptors of the invention, thereby causing the protein to bind to the insoluble matrix of the column. The column is preferably washed one or more times within appropriate buffer solution. The adhesin protein is then eluted using the appropriate agent. This may be free receptor in solution

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or a chaotropic agent, such as KSCN, NaCl, or guanidine hydrochloride. The eluted protein is then tested against the receptors of the invention to confirm that it does bind to them. The purity of the isolated protein may be analyzed by SDS-PAGE.

If desirable, the protein can be further purified by the application of standard protein purification techniques, modified and applied in accordance with the discoveries and teachings described herein. Such techniques include electrophoresis, centrifugation, gel filtration, precipitation, dialysis, chromatography (including ion exchange chromatography, affinity chromatography, immunoadsorbent affinity chromatography, reverse-phase high performance liquid chromatography, and gel permeation high performance liquid chromatography), isoelectric focusing, and variations and combinations thereof. The preferred techniques include those identified and described in U.S. Patent No. 4,446,122 issued May 1, 1984 to Chu, et al., which is incorporated herein by reference. Preferably, the protein is further purified by receptor affinity chromatography.

One or more of these techniques are employed sequentially in a procedure designed to separate molecules according to their physical or chemical characteristics. These characteristics include the hydrophobicity, charge, binding capability, and molecular weight of the protein. The various fractions of materials obtained after each technique are tested for their ability to react with the receptors of the invention. Those fractions showing such activity are then subjected to the next technique in the sequential procedure, and the new fractions are tested

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again. The process is repeated until only one fraction reactive with the receptors remains and that fraction produces only a single band when subjected to polyacrylamide gel electrophoresis.

Thus, the protein of the invention is a substantially pure protein that binds to the receptors of the invention. As used herein, "substantially pure" and related terms mean that the protein has been isolated from other proteins naturally associated with the protein of the invention. The substantially purified protein is at least 80% pure by weight and preferably at least 90% pure by weight. That is, the composition comprising the substantially purified protein contains no more than 20% and preferably no more than 10% by weight of proteins that are not the protein of the invention. Since the protein may be purified further by the application of standard techniques, the invention encompasses the purified protein. As used herein, the term "purified" and variants thereof means that the proteins are at least 95% pure by weight, preferably at least 98% pure by weight, and most preferably at least 99% pure by weight.

The protein of the invention may be modified by known protein modification techniques. These include the techniques disclosed in U.S. Patent No. 4,302,386, issued November 24, 1981 to Stevens, incorporated herein by reference. Such modifications may enhance the immunogenicity or antimicrobial activity of the protein or it may have no effect on such activity.

For example, a few amino acid residues may be changed or removed. Alternatively, the protein of the

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invention may contain one or more amino acid sequences that are not necessary to its immunogenicity or antimicrobial activity. It may be the case, for example, that only the amino acid sequences of a particular epitope of the antigen will be necessary for immunogenic activity. Unwanted sequences can be removed by techniques well-known in the art. For example, unwanted amino acid sequences can be removed via limited proteolytic digestion using enzymes such as trypsin or papain or related proteolytic enzymes.

This approach is expected to be particularly useful for the adhesin protein of the invention. Since the receptors of the invention bind to a broad array of microorganisms, the protein adhesins of those microorganisms may have slight variations in their amino acid sequence. However, they all bind to the consensus sequence GlcNAcB1-3GalB1-4GlcB1-1Cer, whether that sequence is a terminal sequence as in lactotetraosylcer or an internal sequence as in paragloboside. Therefore, such proteins will have a conserved region which is a universal epitope. This universal epitope is the particularly preferred polypeptide of the invention.

Alternatively, polypeptides corresponding to various immunogenic epitopes of the protein may be chemically synthesized by methods well-known in the art. These include the methods disclosed in U.S. Patent No. 4,290,994, issued September 22, 1981 to Goldberg, incorporated herein by reference.

Thus, the protein of the invention includes a class of modified polypeptides, including synthetically derived peptides or fragments of the protein, having common

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elements of origin, structure, and mechanism of action, such as immunogenic or antimicrobial effect or being able to bind to the receptor of the invention, that are within the scope of the present invention because they can be prepared by persons skilled in the art, once given the teachings of the present invention. Moreover, since persons skilled in the art can make modifications to or derivatives of epitopes on the protein of the invention, once such epitopes are identified, such modifications or derivatives are within the scope of the invention, provided that they are immunogenic and have an antimicrobial effect in humans or other animals, particularly including mammals and primates.

Thus, the polypeptide may be an immunogenic fragment of the protein of the invention or a modified polypeptide substantially homologous to this polypeptide. As used herein, the term "substantially homologous" means immunologically cross-reactive. Such a polypeptide may also be identified by the fact that it will bind to antibodies to the adhesin protein of the invention, which antibodies can be prepared by standard techniques.

The adhesin protein of the invention or an immunogenic fragment or polypeptide derived therefrom is expected to have utility as an immunogen in an antimicrobial vaccine for animals, including mammals, primates, and humans. Such a vaccine can be prepared by techniques known to those skilled in the art and would comprise, for example, the antigen, a pharmaceutically acceptable carrier, an appropriate adjuvant, and other materials traditionally found in vaccines. An

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immunologically effective amount of the antigen is determined by means known in the art.

The proteins and polypeptides of the invention may also be used to detect a microorganism that expresses the receptors of the invention. For example, as shown in Figure 3, N. gonorrhoeae was found to contain the receptor GalB1-4GlcNAcB1-3GalB1-4Glc as part of its lipopolysaccharide. Thus, the protein or polypeptide can be attached to a solid support, such as beads or a microtiter well, by standard techniques and employed in methods and kits for the detection of such microorganisms as described previously herein. Similarly, the protein or polypeptide can be attached to a pharmaceutical agent as described previously herein to target such agent to such pathogenic or opportunistic microorganism.

Given the receptors of the invention and the teachings disclosed herein, persons skilled in the art can obtain the DNA that codes for the microbial adhesin polypeptide of the invention in isolated or substantially purified form through the application of standard genetic engineering techniques. Such techniques include those described in Maniatis et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory 1982), incorporated herein by reference.

The DNA of the invention is an isolated or substantially purified DNA sequence (i.e., polydeoxyribonucleotide) encoding a polypeptide that binds to the receptors of the invention. As used herein, the term "isolated" and variations thereof means that the DNA is in isolation from DNA encoding other polypeptides



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normally accompanying this polypeptide. Thus, the DNA of the invention includes DNA encoding the polypeptide when that DNA has been cloned into a microbial vector, such as a plasmid, or into a viral vector that may be harbored by a bacteriophage, provided that such clones are isolated from clones that contain DNA encoding other polypeptides normally accompanying this one. As used herein, the term "substantially pure" and variants thereof means that the DNA is substantially free of DNA and RNA that does not encode the protein of the invention. That is, there will be no more than about 1% by weight of other DNA and RNA and preferably no more than about 0.2% by weight of other DNA and RNA in any sample that contains the DNA of the invention.

Preferably, the DNA is obtained by using the instant receptors to screen an appropriate genomic library that contains the DNA of a pathogenic or opportunistic microorganism. Such a library comprises colonies of a single type of microorganism, generally bacteria like E. coli K12 (HB101), into which pieces of the foreign DNA have been inserted, generally by being incorporated into a plasmid, cosmid, or phage vector compatible with the microorganism. More specifically, the library comprises clones of vectors into which different sequences of the DNA have been operably and recoverably inserted, each of the vectors containing only one sequence of the DNA. The vectors may be plasmids, cosmids, or phage genomes. If necessary because of the type of library being used, segments of DNA will have been inserted into the vectors in a manner that they will be expressed under appropriate conditions (i.e., in proper orientation and correct reading frame and with appropriate expression sequences, including

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an RNA polymerase binding sequence and a ribosomal binding sequence.) The microorganisms will be ones that do not express the adhesin protein, such as E. coli HB101.

Clones from the library are brought into contact with the receptors of the invention to identify those clones that bind to the receptors. Preferably, the library is brought into contact with the receptor on a thin layer chromatography plate to determine which of the microorganisms bind to the receptor. The clones are isolated and the exogenous DNA sequence is recovered from one of the clones. The sequence is evaluated to determine if it encodes the protein.

In a particularly preferred embodiment, the DNA of the invention is obtained by an application and modification of the teachings of Paruchuri, et al., Proc. Natl. Acad. Sci. USA, 87:333-337 (1990), incorporated herein by reference. The authors used a different adhesion receptor for N. gonorrhoeae to identify and characterize the adhesion protein and the gene.

As applied to this invention, a genomic library of N. gonorrhoeae strain MS11 is constructed in the vector pHSS6 (Seifert, et al., Proc. Natl. Acad. Sci. USA, 83:735-739 (1986), incorporated herein by reference) in E. coli HB101 (Seifert, et al., J. Bacteriol., 172:40-46 (1990), incorporated herein by reference. A thin layer chromatography overlay assay is used to identify the clones expressing the adhesion protein. The DNA is then obtained in isolated or substantially purified form.

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In an alternate preferred embodiment, colonies containing DNA that encodes the microbial adhesin gene(s) would be detected using DYNA Beads according to Olsvick et al., 29th ICAAC, Houston, Tex. 1989, incorporated herein by reference. The previously described glycolipids would be crosslinked to tosylated dyna beads M280 and these receptor-containing beads would then be used to adsorb to colonies expressing the adhesin protein(s). Colonies not expressing the adhesin would be removed by washing, and this process would be repeated to obtain an appropriate enrichment. Putative adhesin expressing colonies would then be plated and confirmed by metabolically labeling each colony with 35S-methionine and testing the ability of the colony to bind to the receptor as previously described. The DNA from several adhering clones would be compared to identify shared sequences and these shared sequences would be further subcloned and characterized as previously described.

In another alternate preferred embodiment, the gene(s) for the specific glycolipid adhesin would be localized and identified by constructing a non-adherent mutants of a specific pathogen. This would be accomplished by creating mutants using a transposable element such as TnPhoA as described in Manoil et al., Proc. Natl. Acad. Sci. USA, 82:81129-8133 (1985), incorporated herein by reference. Alkaline phosphatase positive mutants would indicate mutations within exported proteins. Since the adhesin for each pathogen is presumed to be located on the outer membrane surface and therefore exported, this set of mutants would contain a much reduced subset of mutants. They would then be screened for a loss in binding activity by procedures previously described.

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It will be recognized by persons skilled in the art that the DNA sequence for the adhesin protein may be modified by known techniques in view of the teachings disclosed herein. For example, different codons can be substituted that code for the same amino acid as the original codon. Alternatively, the substitute codons may code for a different amino acid that will not affect the immunogenicity or antimicrobial activity of the protein or which may improve its immunogenicity or antimicrobial activity. For example, oligonucleotide directed, site specific mutagenesis or other techniques to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle, "Strategies and Applications of In Vitro Mutagenesis," Science, 229:193-1210 (1985), which is incorporated herein by reference, can be employed. Since such modified DNA can be obtained by the application of known techniques to the teachings contained herein, such DNA is within the scope of the claimed invention.

Moreover, it will be recognized by those skilled in the art that the DNA sequence (or fragments thereof) of the invention can be used to obtain other DNA sequences that hybridize with it under conditions of moderate to high stringency, using general techniques known in the art. Accordingly, the DNA of the invention includes such DNA.

The DNA of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform a microorganism for the expression and production of the polypeptide of the

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invention. Such techniques include those disclosed in U.S. Patent Nos. 4,440,859 issued April 3, 1984 to Rutter et al., 4,530,901 issued July 23, 1985 to Weissman, 4,582,800 issued April 15, 1986 to Crowl, 4,677,063 issued June 30, 1987 to Mark et al., 4,678,751 issued July 7, 1987 to Goeddel, 4,704,362 issued November 3, 1987 to Itakura et al., 4,710,463 issued December 1, 1987 to Murray, 4,757,006 issued July 12, 1988 to Toole, Jr., et al., 4,766,075 issued August 23, 1988 to Goeddel, et al., and 4,810,648 issued March 7, 1989 to Stalker, all of which are incorporated herein by reference.

The DNA of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host cell. The companion DNA would depend upon the nature of the host cell, the manner of the introduction of the DNA into the host cell, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognized by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. Once selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such

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as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell. The preferred expression vector for use in the invention is the Bluescript series from Stratogene, Inc. The preferred host cell is DH5 alpha.

The transformed host cells express the proteins or polypeptides of the invention. Such cells are cultured by known techniques, and the proteins or polypeptides are recovered by known techniques. Depending upon the host and expression system used, the recombinant proteins and polypeptides of the invention may be part of a fusion protein produced by the transformed host cells. Such proteins are recovered by known techniques, and the undesired part may be removed by known techniques. Alternatively, the fusion protein itself may be more immunogenic than the recombinant protein or polypeptide alone and, therefore, may itself be used in a vaccine.

In a particularly preferred embodiment, the vaccine of the invention comprises an avirulent microorganism that has been transformed by the DNA of the invention, whereby the transformed microorganism expresses the protein or polypeptides of the invention in a manner such that the delivery of the avirulent transformed microorganism to an animal or human host results in an immune response. Generally, the foreign antigenic determinants are expressed on the surface of the microorganism. Preferably, the microorganism is in a pharmaceutically acceptable carrier. Particularly preferred microorganisms would be species of Salmonella, which can be prepared for the oral delivery of heterologous antigens according to the methods disclosed in

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Chatfield, et al., Vaccine, 7:495-498 (1989), incorporated herein by reference.

It is to be understood that the application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and processes for their use appear in the following examples.

#### EXAMPLE 1

##### Glycolipid Adhesion Receptors for *N. gonorrhoeae*

This Example shows that lacto-series glycolipids containing the minimum carbohydrate sequence GlcNAcB1-3GalB1-4Glc can support adhesion of *N. gonorrhoeae* and that binding is not dependent on pili, protein II (P II), or the presence of lipooligosaccharide (LOS). Interestingly, lacto-series structures are also found in some LOS derived from the outer membrane of *N. gonorrhoeae* (15), which may explain the well-known phenomenon of autogglutination seen with this organism.

#### Experimental Procedures

Materials. Gonococcal LOS was isolated from acetone-powdered organisms by the hot-phenol method (16,17). Murine IgG monoclonal antibody 4BE12 against meningococcal LOS serotype 3,7,9(18) was provided by Dr. Wendell Zollinger (Walter Reed Army Institute of Research, Washington, D.C.) Affinity-purified goat

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anti-mouse IgM (Kirkegaard and Perry, Gaithersburg, MD) was labeled with  $^{125}\text{I}$  (ICN Biomedicals, Costa Mesa, CA) by the Iodo-Gen method (19) to a specific activity of approximately 25 Ci/ug. (As used in the context of measurements in the examples of this application, u equals micro.) Bovine testes B-galactosidase, neuraminidase (Arthrobacter ureafaciens) and bovine serum albumin (BSA, fraction V) were purchased from Boehringer Mannheim (Indianapolis, IN) N-Acetyl-B-D-hexosaminidase was from Genzyme (Boston, MA). All standard ganglioside and neutral glycolipids were from BioCarb Chemicals (Lung, Sweden). Aluminum-backed silica gel high performance thin-layer chromatography plates were purchased from Merck (West Germany). Alpha-2-3Sialyllacto-neotetraosylceramide (sialylparagloboside) was isolated from type O human erythrocytes (20). Lacto-N-neotetraosylceramide (paragloboside) was prepared by desialylation of erythrocyte sialylparagloboside with 1 M formic acid for 60 min at 100° C. Lacto-N-triaosylceramide was prepared by digestion of paragloboside with B-galactosidase as described for the preparation of asialo-GM2 from asialo-GM1 (14). The concentrations of glycolipids listed in Table I were determined by densitometry (Quick-scan, Helena Laboratories) of orcinol-stained thin-layer chromatograms compared with authentic standards. The purity of all lipids was confirmed by thin-layer chromatography in neutral and acidic solvent systems.

Growth and Labeling of *N. gonorrhoeae*. The gonococcal strains and variants are described in Table II. The microorganisms were grown in supplemented GCB (Difco) broth with shaking at 1500 rpm or on agar plates at 37° C in 5% CO<sub>2</sub>/95% air. Cells grown anaerobically on GCB agar



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were supplemented with nitrate, as described (21). Gonococci grown under iron-limited conditions was done by passing the cells three times on media supplemented with 25 uM Desferal (Ciba-Geigy), as described (22). Microorganisms were scraped from agar plates, or harvested from both by centrifugation, and suspended in 0.01 M sodium phosphate, pH 7.4, containing 0.15 M sodium phosphate (PBS). Microorganisms were centrifuged at 4° C and 10,000 x g and pellets were washed twice in PBS. The cells were radioiodinated as described (23) using 0.4 mCi of Na<sup>125</sup>I (ICN Biomedicals). The iodination was terminated after 3 min by removing the cells from the reaction tube, followed by centrifugation and two washes in PBS. The labels gonococci were resuspended to 2.5 x 10<sup>6</sup> or 4 x 10<sup>6</sup> cpm/ml in Hanks Balanced Salt Solution, pH 7.4, containing 1% bovine serum albumin (HBSS-BSA).

#### Assay of N. gonorrhoeae Binding to Glycolipids.

Gonococci bound to glycolipids separated by high performance thin-layer chromatography were detected as described (23). The binding of gonococci to purified glycolipids immobilized in microtiter plates (Falcon 3912-III, Bectin-Dickinson) was measured as previously described (23).

Immunostaining Glycolipids with Anti-LOS Monoclonal Antibody. Authentic paragloboside was detected on thin-layer chromatograms by immunostaining followed by autoradiography for 24 hours (24).

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## Results and Discussion

Binding of *N. gonorrhoeae* to Lacto-Series Glycolipids on Chromatograms. The glycolipid specificity of the different gonococcal isogenic variants was established by the thin-layer overlay assay and is summarized in Table II. As shown by an autoradiogram (Fig. 1B) compared with an identical thin-layer plate visualized with orcinol reagent (Fig. 1A), *N. gonorrhoeae* binds avidly to authentic paragloboside and the product obtained after B-galactosidase treatment, lacto-N-triaosylceramide (Fig. 1B, lanes 3 and 4). No binding was detected to lactosylceramide derived from lacto-N-triaosylceramide after treatment with N-acetyl-B-hexosaminidase (Fig. 1B, lane 5), or to the other neutral glycolipids tested (Fig. 1B, lane 1; Table I). These data appear to be in variance with those of Stromberg et al. (12) who reported that *N. gonorrhoeae* does not bind to lacto-N-triaosylceramide and lacto-N-neotetraosylceramide (paragloboside). Possibly, this discrepancy is related to the source and/or fatty acid contents (12) of the glycolipids used by these investigators. In some experiments, *N. gonorrhoeae* bound weakly to some of the gangliosides (Table I), including sialylparagloboside (Fig. 1B, lane 2) and GM1 (Fig. 1B, lane 1); however, this observation was not always reproducible, and none of the gangliosides demonstrated concentration-dependent binding in microtiter plates (Fig. 2).

Quantitative Binding of *N. gonorrhoeae* to Immobilized Glycolipids and Inhibition of Binding by Glycolipids. Binding of *N. gonorrhoeae* to purified

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glycolipids adsorbed on microtiter plates was examined to further define the binding specificity and to compare the relative avidities of the lacto-and ganglio-series receptors. As shown in Fig. 2, the gonococci bind better to asialo-GM2 than to paragloboside and not at all to sialyparagloboside, GM2 is half-maximal at 0.2 ug, about 7 times better than to paragloboside (or lacto--N-triaosylceramide, data not shown), suggesting a higher avidity for the ganglio-series glycolipids. Binding of P+ and P- variants of *N. gonorrhoeae* to both asialo-GM1 and paragloboside was not affected by changing the growth conditions of the organism, as the gonococci bound equally well to both glycolipids when grown anaerobically, microaerophilically on agar or in broth, or under iron limited conditions (data not shown).

**Biological Significance.** *N. gonorrhoeae* agglutinate human erythrocytes (25) and adhere to and are phagocytosed by neutrophils (26). The receptor that mediates binding of these cells to the gonococci is probably paragloboside and lacto-N-triaosylceramide which are present in substantial amounts in both cell types (14,16,27). Lacto-series also constitute the major glycolipid component of various tissues and organs and are glycolipid precursors of the major blood group antigens. Interestingly, GalB1-4GlcNAcB1-4Glc... sequences are also present in may gonococcal LOS (15). As shown in Fig. 3, an anti-LOS antibody (4BE12) (18) binds strongly to authentic human paragloboside, detecting as little as 30 ng of glycolipid. Gonococcal lipopolysaccharide which contains the 4.8 kD LOS component is bound by monoclonal antibody 4BE12 thus confirming the presence of the GalB1-4GlcNAcB1-4GalB1-4Glc... sequence in this lipopolysaccharide (data

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not shown). This LOS also strongly inhibits gonococcal specific agglutination of human erythrocytes, as well as inhibits the binding of radiolabeled P+ and P- gonococci to paragloboside and lacto-N-triaosylceramide on thin-layer chromatograms (data not shown). Thus, the mechanism for the well-known phenomenon of gonococcal autoagglutination (29,30) may be explained by an adhesin of one organism binding to GlcNAcB1-3GalB1... sequences in the LOS of another organism. As microorganisms' LOS do not contain ceramide, binding of *N. gonorrhoea* to at least the lacto-series glycolipids is probably not dependent on the fatty acid in ceramide as has been reported for other microorganisms (31).

Asialo-GM2 has been reported to occur in cultured human endocervical cells (12), a relevant target tissue for infection, and asialo-gangliosides also occur in other human tissues, although in lower amounts (14,32,33). Both asialo-GM1 and asialo-GM2, however, bind *N. gonorrhoeae* with highest avidity (Fig. 2). Recently, Paruchuri et al. (34) have identified the gene encoding for an adhesin which binds to asialo-GM1 and asialo-GM2 and have shown that the adhesin is 36 kD protein that is not associated with gonococcal pili. As mutants that do not express this adhesin retain their ability to agglutinate human erythrocytes (34), this adhesin probably is distinct from the paragloboside-binding specificity we describe here. Thus, more than one type of gonococcal adhesin may mediate binding to different human cell types and individually or coordinately contribute to the pathogenesis of disease.

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Summary

The role glycolipids as adhesion receptors for Neisseria gonorrhoeae was examined. Serum resistant isolates, piliated and nonpiliated isogenic variants, as well as gonococci deficient in lipooligosaccharide (LOS) and protein II, bound specifically to terminal and internal GlcNAcB1-3GalB1-4Glc sequences in lacto-series glycolipids, as measured by overlaying glycolipid chromatograms with <sup>125</sup>I-labeled organisms. The binding activity was not affected by changing the growth condition of the organism, as the gonococci bound to the glycolipids when grown anaerobically, microaerophilically on agar or in broth, or under iron-limited conditions. The gonococci did not bind to lactosylceramide (GalB1-4GlcB1-1Cer) derived from lacto-N-triaosylceramide or from asialo-GM2 by treatment with N-acetyl-B-hexosaminidase, or to other neutral glycolipids tested. Although N. gonorrhoeae bound weakly to some gangliosides on thin-layer chromatograms, including sialylparagloboside and GM1, in solid phase assays the gonococci bound with moderate avidity to the sequence GlcNAcB1-3GalB1-4Glc and not at all to gangliosides. Interestingly, the 4.8 kD component of gonococcal LOS, which contains lacto-N-neotetraose (GalB1-4GlcNAcB1-3GalB1-4Glc), strongly inhibited gonococcal specific agglutination of human erythrocytes and inhibited the binding of labeled organisms to human paragloboside and lacto-N-triaosylceramide on thin-layer chromatograms. Possibly, this binding specificity explains why gonococci autoagglutinate in vitro.

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EXAMPLE 2Adhesion Receptors for Other Microorganisms

A wide variety of other microorganisms were tested according to the methods of Example 1 to determine their ability to bind to the receptors of the invention. Those organisms are identified in Table III. It was determined that such microorganisms bound to the receptor with the same range of affinity as the N. gonorrhoeae bound the receptors in Example 1. Figure 4 is a representative example, showing the binding of Pseudomonas aeruginosa to paragloboside.

EXAMPLE 3Purification of Bacterial Adhesin Protein

This example shows how to obtain the purified bacterial adhesin protein that binds to the receptors of the invention. The steps are as follows:

1. Growth of organism. A special defined medium, low methionine, is prepared, and 75ml of this fresh media in a 100ml glass sterile bottle is inoculated with the organism. Then 0.5 mCi of <sup>35</sup>S-methionine is added. The organism is grown at 37°C, 5% CO<sub>2</sub> for 24 hrs.

2. Harvest of organism. After 24 hrs. the organism is centrifuged 10,000 x g for 10 min. at 4°C. The pellet is resuspended in 20 ml of 10mM Hepes pH 7.4, saline and put on ice.

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3. Preparation of Omeps. The resuspended organism is sonicated 6 times on ice for 30 seconds each at a setting of 4 on the Bronson Sonicator. Then the disrupted organism is centrifuged 10,000 x g for 10 min. at 4°C. The supernatant is centrifuged 100,000 x g for 30 min. at 4°C. The resulting pellet is called Omeps. Protease inhibitors are added (PlC 1 + 11) and stored for 2 weeks at 4°C.

4. Solubilization of Omeps. Omeps are centrifuged 100,000 x g as above. The pellet is resuspended in 4 ml of 10mM Hepes pH 8.0, 1.3% Octyl-Glucopyranoside (Sigma), sonicated for 5 minutes, and incubated at room temperature for 30 minutes. Then the Omeps are centrifuged at 100,000 xg as before. The resulting supernatant contains soluble adhesin.

5. Purification of Adhesin Protein. The adhesin is purified from the octylglucopuroanoside supernatant by diluting it 1/10 in 50mM Tris-HCl pH 7.8, 150mM NaCl, 1.0% BSA and incubating it in previously blocked wells of an ELISA plate containing 0.8 ug/well of receptor for 2 hrs. at room temperature. Wells are washed 4X with cold normal saline, then 30 ul of 60°C 10mM Tris-HCl, pH 8.0, saline, 0.1% SDS is added to each well and incubated for 30 min. at 37°C. Alternatively, the adhesin is purified by receptor affinity column chromatography, where the receptor is immobilized as an insoluble support like agarose beads. The SDS elution buffer is removed from appropriate wells and analyzed on SDS-PAGE and autoradiography.

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TABLE I

Glycolipids Tested for Ability to Bind H. gonorrhoeae on  
Thin-Layer Chromatograms

Glycolipid <sup>a</sup>	Structure	Binding <sup>b</sup>
Asialo-GM2	GalNAcB1-4GalB1-4GlcB1-1Cer	++
Asialo-GM1	GalB1-3GalNAcB1-4GalB1-4GlcB1-1Cer	++
Lactotriaosylcer	GlcNAcB1-3GalB1-4GlcB1-1Cer	+
Paragloboside	GalB1-4GlcNAcB1-3GalB1-4GlcB1-1Cer	+
Glucosylcer (CMH)	GlcB1-1Cer	-
Lactosylcer (CDH)	GalB1-4B1c-1Cer	-
Trihexosylcer (CTH)	Gala1-4GalB1-GlcB1-1Cer	-
Globoside (GL4)	GalNAcB1-3Gala1-4GalB1-4GlcB1-1Cer	-
Forssman (Fors)	GalNAcA1-3GalNAcB1-3Gala1-4GalB1-4GlcB1-1Cer	-
GM3	NeuAca2-3GalB1-4GlcB1-1Cer	-
GM2	GalNAcB1-4[NeuAca2-3]GalB1-4GlcB1-1Cer	-
GM1	GalB1-3GalNAcB1-4[NeuAca2-3]GalB1-4GlcB1-1Cer	+/-
Sialylparagloboside	NeuAcaGalB1-4GlcNAcB1-3GalB1-4GlcB1-1Cer	+/-
GD3	NeuAca2-8NeuAca2-3GalB1-4GlcB1-1Cer	+/-
GD1a	NeuAca2-3GalB1-3GalNAcB1-4[NeuAca2-3]GalB1-4GlcB1-1Cer	+/-
GD1b	GalB1-3GalNAcB1-4[NeuAca2-8NeuAca2-3]GalB1-4GlcB1-1Cer	+/-
GT1b	NeuAca2-3GalB1-3GalNAcB1-4[NeuAca2-8NeuAca2-3]GalB1-4GlcB1-1Cer	-

<sup>a</sup> Trivial names and structures are represented according to recommendations in Ref 38 and references cited therein; cer, ceramide; CMH, ceramide monohexoside; CDH, ceramide dihexoside (lactosylceramide); CTH, ceramide trihexoside; GL4, globoside.

<sup>b</sup> Negative binding (-) indicates no binding to 2 ug of glycolipid and positive binding to less than 0.4 ug (++) , 0.8-1 ug(+), and 1 ug(+/-).



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TABLE II

N. gonorrhoeae Strains Used in This Study and Their Glycolipid Binding Specificities<sup>a</sup>

Strain (Ref.)	Phenotype <sup>b</sup>	<u>Binding to</u>	
		Asialo-GM1	Paragloboside
NS11 <sub>mk</sub> (34)	P+, PII-	++	+
NS11 <sub>mk</sub> (34)	P-, PII-	++	+
FA5100 (35)	LOS-/MAB unreactive	++	+
WG-52 (36)	serum resistant	++	+

<sup>a</sup> Determined by the microorganism overlay assay using 0.5 ug of purified asialo-GM1 or 1 ug paragloboside as described in Materials and Methods.

<sup>b</sup> Abbreviations used: P+, pilliated; P-, nonpilliated; PII, protein II deficient; LOS-, Lipooligosacchahride deficient; MAB, monoclonal antibody.

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TABLE III

Other Pathogenic and Opportunistic Microorganisms That  
Bind to the Receptors Lactotriaoylcer and Paragloboside

---

<u>Streptococcus pneumoniae</u>	<u>Neisseria gonorrhoeae</u>
<u>Streptococcus agalactiae</u> (Gp.B)	<u>Neisseria meningitidis</u>
<u>Staphylococcus aureus</u>	<u>Shigella dysenteriae</u>
<u>Clostridium perfringens</u>	<u>Shigella flexneri</u>
<u>Borrelia burgdorferi</u> (Lyme agent)	<u>Coxiella burnettii</u>
<u>Clostridium difficile</u>	<u>Pseudomonas aeruginosa</u>
<u>Haemophilus influenzae</u>	<u>Pseudomonas cepacia</u>
<u>Haemophilus parainfluenzae</u>	<u>Pseudomonas maltophilia</u>

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WE CLAIM:

1. A receptor for a pathogenic or opportunistic microorganism comprising a substantially pure compound selected from the group consisting of GalB1-4GlcNAcB1-3GalB1-4GlcB1-1-X(R), GalB1-3GlcNAcB1-3GalB1-4GlcB1-1-X(R), GlcNAcB1-3GalB1-4GlcB1-1-X(R), GalB1-4GlcNAcB1-3GalB1-4Glc, GalB1-3GlcNAcB1-3GalB1-4Glc, GlcNAcB1-3GalB1-4Glc, GalB1-4GlcNAcB1-3Gal, and GalB1-3-GlcNAcB1-3Gal wherein X is sphingosine, hydroxylated sphingosine, or saturated sphingosine and R is H or an N-acyl fatty acid derivative of X such that X(R) is a ceramide.
2. The receptor of claim 1 wherein said pathogenic or opportunistic microorganism is selected from the group consisting of Streptococcus, Staphylococcus, Clostridium, Borrelia, Haemophilus, Pseudomonas, Neisseria, Coxiella, Shigella, and Rotavirus.
3. The receptor of claim 1 wherein said substantially pure compound further comprises GlcNAcB1-3GalB1-4Glc and said pathogenic or opportunistic microorganism is selected from the group consisting of Streptococcus agalactiae (Gp. B), Staphylococcus aureus, Clostridium perfringens, Clostridium difficile, Borrelia burgdorferi, Haemophilus influenzae, Haemophilus parainfluenzae, Pseudomonas aeruginosa, Pseudomonas cepacia, Pseudomonas maltophilia, Neisseria gonorrhoeae, Neisseria meningitidis, Coxiella burnetti, Shigella dysenteriae, and Shigella flexneri.

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4. A composition useful as a receptor for pathogenic or opportunistic microorganisms comprising the receptor of claim 1 attached to an insoluble or soluble substrate.

5. The composition of claim 4 wherein said insoluble substrate is a bead.

6. The composition of claim 4 wherein said receptor is attached to a liposome.

7. The composition of claim 4 wherein said soluble substrate is a polypeptide.

8. A method for detecting the presence of pathogenic or opportunistic microorganisms in a sample suspected of containing said microorganisms comprising the steps of:

contacting said sample with the receptors of claim 1 for a period of time and under conditions sufficient for said receptors to bind to said microorganisms if said microorganisms are in said sample; and

determining if said receptors have bound to said microorganisms.

9. The method of claim 8 wherein said receptor is attached to an insoluble substrate.

10. The method of claim 9 wherein said step of determining if said receptor has bound to said microorganisms comprises the steps of:



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contacting any receptor-microorganism complexes formed by the method of claim 9 with an antibody to an antigen on the surface of said microorganisms, wherein said antibody is labeled with a detectable moiety, thereby forming receptor-microorganism-antibody complexes;

removing unbound antibody; and

detecting the presence of said detectable moiety.

11. A diagnostic kit for the detection of pathogenic or opportunistic microorganisms in a sample comprising, in a container:

the receptor of claim 1 attached to an insoluble substrate; and

means for detecting or measuring the formation of complexes of said microorganisms and said receptors.

12. The kit of claim 11 wherein said insoluble substrate is the well of a microtiter plate.

13. The kit of claim 11 wherein said detecting or measuring means is a reagent containing a detectable moiety and capable of binding to said microorganisms.

14. The kit of claim 13 wherein said reagent containing a detectable moiety is the receptor of claim 1 conjugated with a detectable moiety.

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15. The kit of claim 13 wherein said reagent is an antibody to a surface antigen of said microorganisms conjugated to an enzyme.

16. The kit of claim 11 wherein said detecting or measuring means comprises a first antibody to a surface antigen of said bacterium and a second antibody to said first antibody, wherein said second antibody is conjugated to a detectable moiety.

17. The kit of claim 11 wherein said detecting or measuring means is an immunoassay selected from the group consisting of radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and immunofluorescent assay.

18. A method for removing pathogenic or opportunistic microorganisms from a liquid comprising the steps of:

contacting said liquid with the receptors of claim 1 for a period of time and under conditions sufficient to bind said microorganisms to said receptors; and

removing said liquid from contact with said receptors.

19. The method of claim 18 wherein said receptor is attached to a solid support.

20. An apparatus for removing pathogenic or opportunistic microorganisms from a liquid comprising:

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container means for holding said liquid, said container means having an inlet for introducing said liquid into said container means and an outlet for removing said liquid from said container means; and

the receptor of claim 1 within said container means.

21. The apparatus of claim 20 wherein the receptor of claim 1 is bound to the surface of said container means that is exposed to said liquid.

22. The apparatus of claim 20 wherein said receptor is bound to a solid support within said container means.

23. The apparatus of claim 22 wherein said solid support is an insoluble matrix and said container means is a chromatographic column.

24. A pharmaceutical composition for the treatment, prevention, or amelioration of illness or infection in mammals caused by pathogenic or opportunistic microorganisms comprising an effective amount of the receptor of claim 1 in combination with a pharmaceutically acceptable carrier.

25. The composition of claim 24 wherein said carrier is a liposome.

26. The composition of claim 24 wherein said receptor is coupled to a macromolecular carrier.

27. The composition of claim 26 wherein said macromolecular carrier is a polymer.

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28. The composition of claim 24 wherein said receptor is bound to an antimicrobial agent or to an immunogen.

29. A method for treating, preventing, or ameliorating illness or infection in a mammalian host caused by pathogenic or opportunistic microorganisms comprising administering an effective amount of the composition of claim 24.

30. A method of inhibiting the adherence of pathogenic or opportunistic microorganisms to mammalian cells or removing said microorganisms from said cells comprising providing a sufficient amount of the receptor of claim 1 to said cells to inhibit the adherence of said microorganisms or remove said microorganisms.

31. The method of claim 30 wherein said receptor is dissolved or suspended in a liquid which is physiologically compatible with said cells.

32. A method for producing an isolated microbial adhesin protein comprising the steps of:

solubilizing the membranes of pathogenic or opportunistic microorganisms, thereby producing solubilized material containing said adhesin protein and insoluble material;

separating the solubilized material from the insoluble material;

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contacting said solubilized material with the receptor of claim 1, wherein said receptor is attached to an insoluble solid support, for a period of time sufficient for said adhesin protein to bind to said receptor; and

removing said protein from said receptor, thereby recovering said protein in isolated form.

33. The isolated microbial adhesin protein produced by the process of claim 32.

34. A substantially purified microbial adhesin protein, obtained from the surface of a pathogenic or opportunistic microorganism, that binds to the receptor of claim 1.

35. A substantially purified microbial adhesin protein, obtained from the surface of a pathogenic or opportunistic microorganism, that binds to the receptor of claim 3.

36. A substantially pure polypeptide capable of eliciting an antigenic response in an animal host, said antigenic response preventing or ameliorating disease or infection in said host caused by pathogenic or opportunistic microorganisms, wherein said polypeptide is a fragment of the protein of claim 34.

37. An immunogenic polypeptide immunologically cross-reactive with the polypeptide of claim 34.

38. The polypeptide of claim 37 wherein said polypeptide is altered to enhance its immunogenicity.

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39. A substantially pure polypeptide that binds to antibodies to the protein of claim 34.

40. A vaccine comprising an immunologically effective amount of the polypeptide of claim 34 in a pharmaceutically acceptable carrier.

41. A diagnostic kit for the detection in a sample of a pathogenic or opportunistic microorganism that expresses the receptor of claim 1 on its surface comprising, in a container:

the protein of claim 34 attached to an insoluble substrate; and

means for the detecting or measuring the formation of complexes of said protein and said microorganism.

42. A pharmaceutical composition for the treatment, prevention, or amelioration of disease or infection in mammals caused by a pathogenic or opportunistic microorganism that expresses the receptor of claim 1 on its surface comprising an effective amount of the protein of claim 34 in a pharmaceutically acceptable carrier.

43. A method for producing an isolated or substantially purified DNA sequence encoding a microbial adhesin protein comprising the steps of:

screening a genomic library containing the DNA of a pathogenic or opportunistic microorganism, said library comprising clones of vectors into which different sequences

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of said DNA have been operably and recoverably inserted, each of said vectors containing only one sequence of said DNA, by contacting the clones comprising said library with the receptor of claim 1 to identify a clone that binds to said receptors; and

isolating said clone.

44. The method of claim 43 further comprising the steps of:

recovering the exogenous DNA sequence from said clone; and

evaluating said DNA sequence to determine if it encodes said protein.

45. The isolated DNA sequence produced by the process of claim 43.

46. An isolated or substantially purified DNA sequence encoding a microbial adhesin protein that binds to the receptor of claim 1.

47. An isolated or substantially purified DNA sequence derived from the DNA sequence of claim 45 by single or multiple mutations.

48. A DNA sequence that hybridizes with the DNA sequence of claim 45 under conditions of moderate stringency.

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49. A recombinant DNA sequence comprising the DNA sequence of claim 46 operably linked to appropriate regulatory control nucleic acid sequences that are capable of effecting the expression of said DNA sequence in transformed host cells.

50. An expression vector for expressing DNA that codes for the protein of claim 34 in a compatible host comprising an expression vector capable of transforming a procaryotic or eucaryotic cell and the DNA of claim 46 inserted into said vector in proper orientation and correct reading frame for expression.

51. A host cell transformed with the recombinant DNA sequence of claim 46.

52. The recombinant protein produced by the transformed cell of claim 51.

53. A method for producing the protein of claim 34 comprising the steps of:

culturing host cells transformed by a recombinant DNA sequence comprising a DNA sequence that codes for the protein of claim 34 operably linked to appropriate regulatory control nucleic acid sequences that are capable of effecting the expression of said DNA sequence in said transformed cells; and

recovering the polypeptide whose expression has been coded for by said sequence.



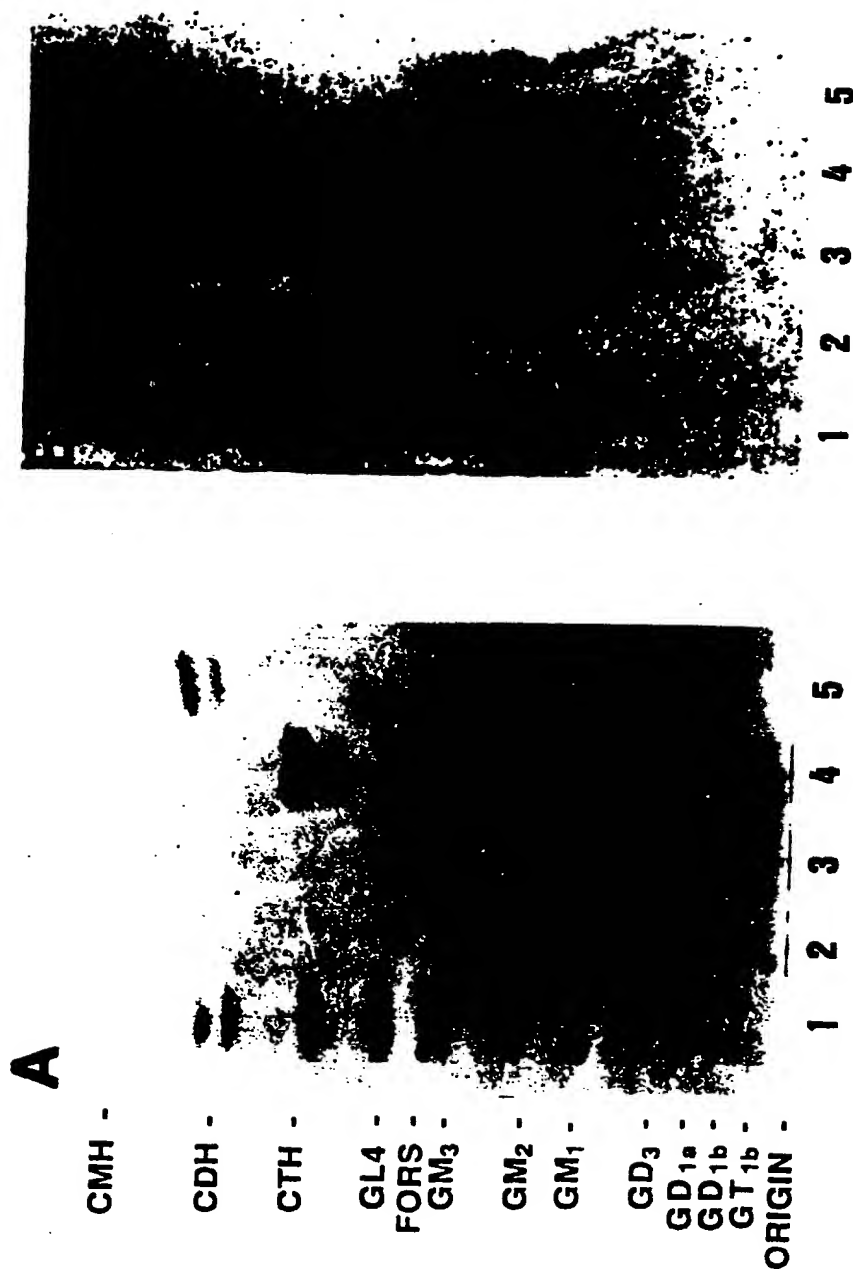
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54. An immunogenic polypeptide comprising a fusion protein containing the protein of claim 34.

55. A vaccine comprising an immunologically effective amount of the recombinant protein of claim 52 in a pharmaceutically acceptable carrier.

56. A vaccine comprising an immunologically effective amount of an avirulent microorganism transformed by the DNA of claim 49, wherein said microorganism is in a pharmaceutically acceptable carrier.

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**FIG. 1A**



**FIG. 1B**

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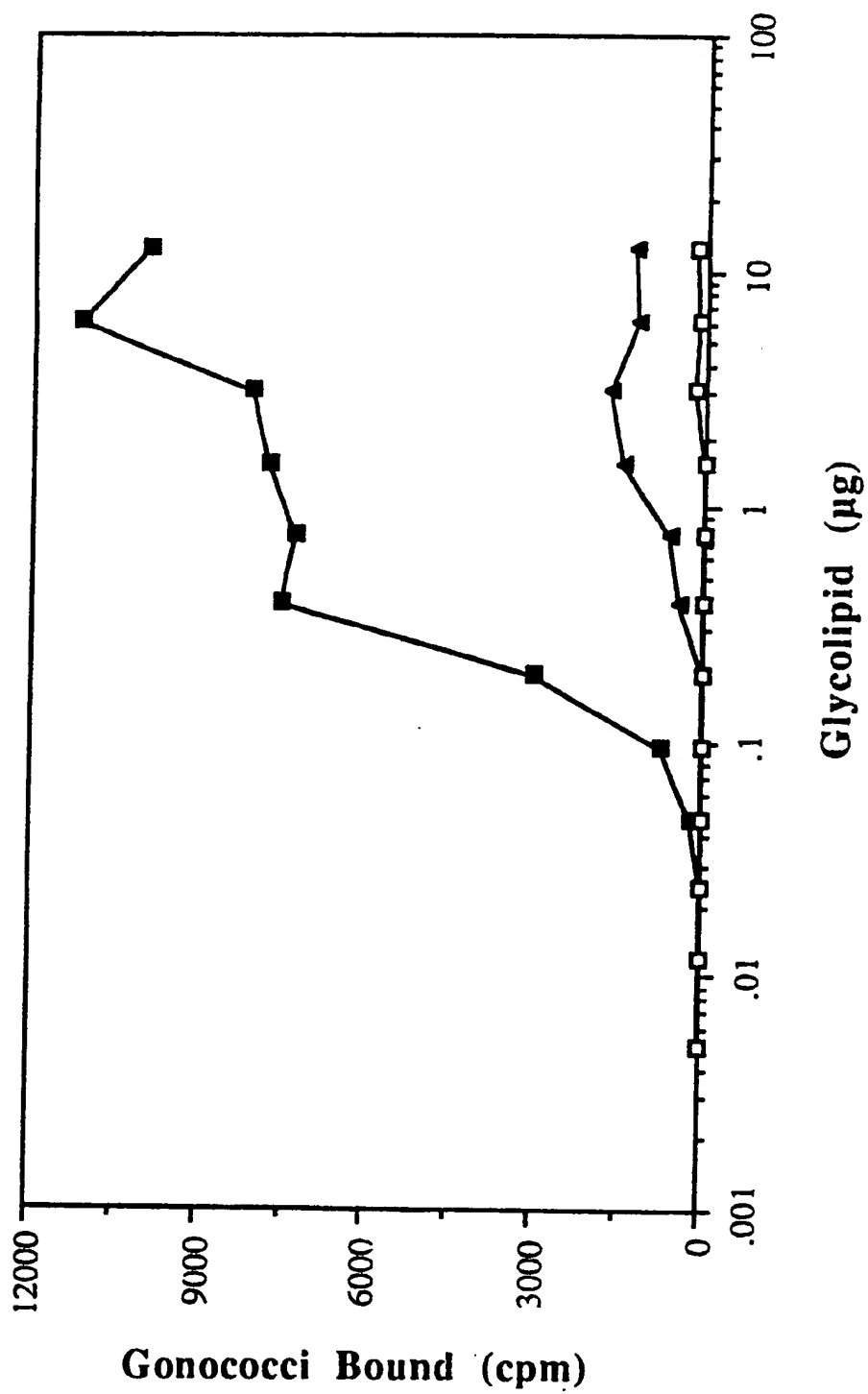


FIG. 2

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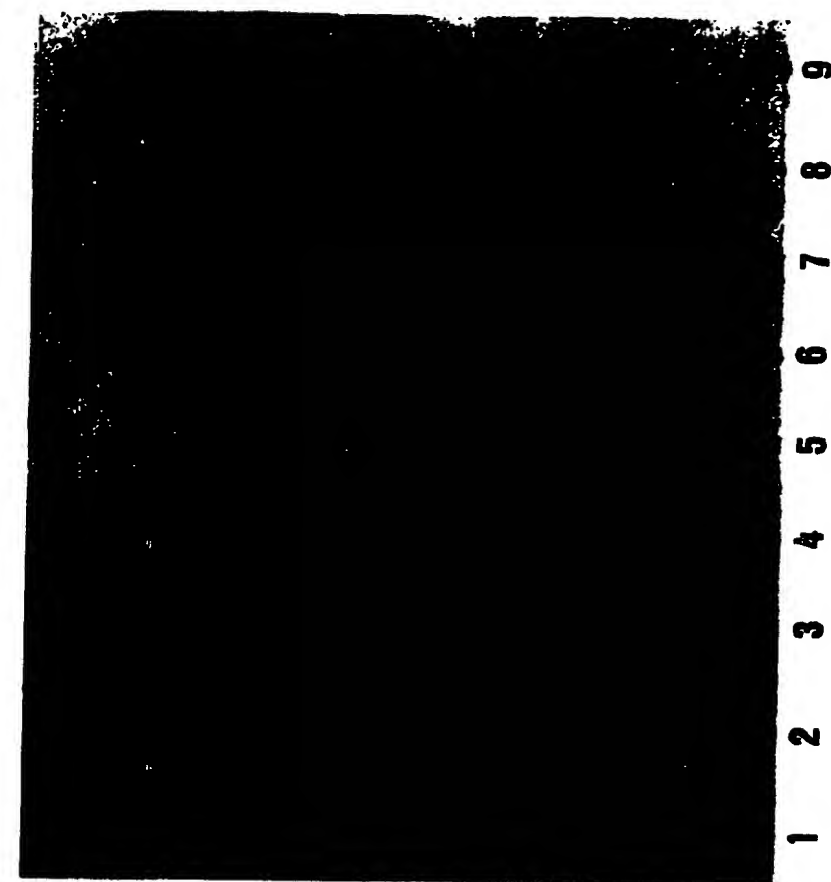


FIG. 3B

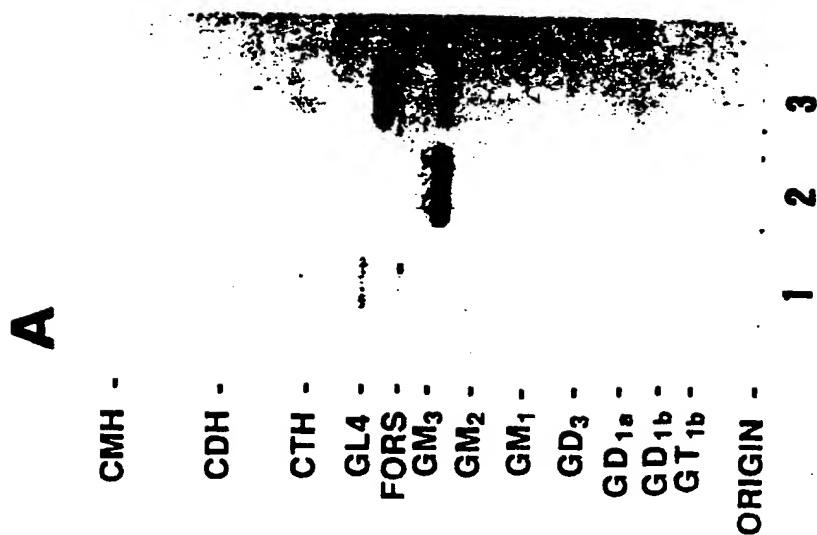
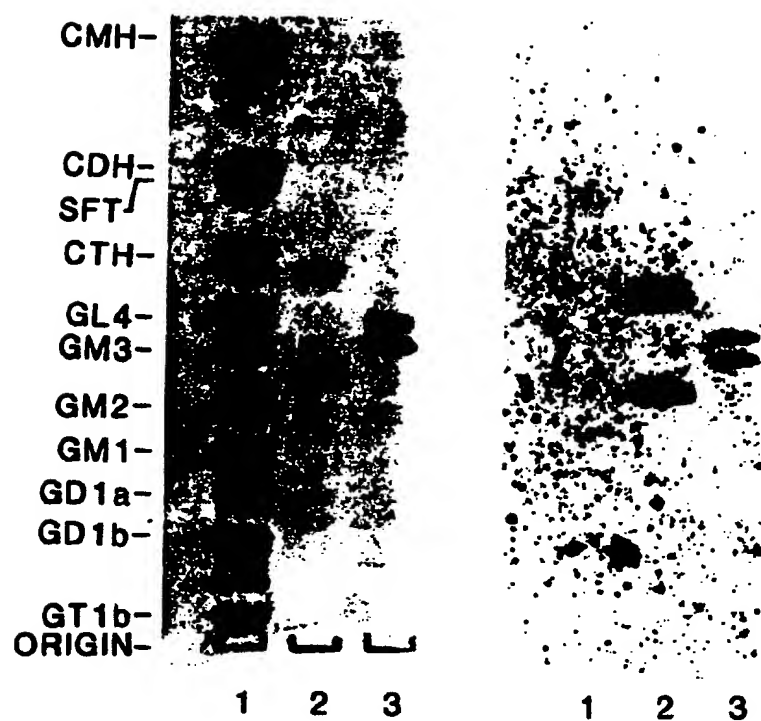


FIG. 3A

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**FIG. 4A** **FIG. 4B**

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05179

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5):G01N 33/53; C12P 21/00 U.S. CL.: 435/7.2, 69.3											
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched 7</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">U.S.</td> <td style="padding: 5px;">424/9,488,493,499,87; 435/6,7,17.2,7.32,7.8,7.92,14,29,69.1 71.1,91,172.3,174,243,261,810,961,971,975; 436/501,518,529,543,586,811. P29;514/2,62;436/71;536/27;530/387;935/9,19,50,54,72,78; 530/810,820</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *</div> <p style="padding: 5px;">Dialog Databases in Biotechnology</p>			Classification System	Classification Symbols	U.S.	424/9,488,493,499,87; 435/6,7,17.2,7.32,7.8,7.92,14,29,69.1 71.1,91,172.3,174,243,261,810,961,971,975; 436/501,518,529,543,586,811. P29;514/2,62;436/71;536/27;530/387;935/9,19,50,54,72,78; 530/810,820					
Classification System	Classification Symbols										
U.S.	424/9,488,493,499,87; 435/6,7,17.2,7.32,7.8,7.92,14,29,69.1 71.1,91,172.3,174,243,261,810,961,971,975; 436/501,518,529,543,586,811. P29;514/2,62;436/71;536/27;530/387;935/9,19,50,54,72,78; 530/810,820										
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 5px;">Category *</th> <th style="width: 70%; padding: 5px;">Citation of Document, 11 with indication, where appropriate, of the relevant passages 12</th> <th style="width: 20%; padding: 5px;">Relevant to Claim No. 13</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">Journal of Experimental Medicine, Volume 168, issued July 1988, E. Tuomanen et al., "Receptor Analogs and Monoclonal Antibodies that Inhibit Adherence of <u>Bordetella Pertussis</u> to Human Citrate Respiratory Epithelial Cells," pages 267-277, see especially the "Materials and Methods" section on pages 268-270.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-4,8-11 13,15-17 24,26-29 32-42</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">Proceedings of the National Academy of Sciences, Volume 86, issued June 1989, Haltgren et al., "The Pap G adhesion of uropathogenic <u>Escherichia coli</u> contains separate regions for receptors binding and for the incorporation into pilus", pages 4357-4361, see especially the "MATERIALS AND METHODS" section on pages 4357-4358.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">32-52, 54-56</td> </tr> </tbody> </table>			Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13	Y	Journal of Experimental Medicine, Volume 168, issued July 1988, E. Tuomanen et al., "Receptor Analogs and Monoclonal Antibodies that Inhibit Adherence of <u>Bordetella Pertussis</u> to Human Citrate Respiratory Epithelial Cells," pages 267-277, see especially the "Materials and Methods" section on pages 268-270.	1-4,8-11 13,15-17 24,26-29 32-42	Y	Proceedings of the National Academy of Sciences, Volume 86, issued June 1989, Haltgren et al., "The Pap G adhesion of uropathogenic <u>Escherichia coli</u> contains separate regions for receptors binding and for the incorporation into pilus", pages 4357-4361, see especially the "MATERIALS AND METHODS" section on pages 4357-4358.	32-52, 54-56
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: 14</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>											
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;">           Date of the Actual Completion of the International Search  <div style="border: 1px solid black; padding: 2px; display: inline-block;">25 November 1991</div> </td> <td style="width: 50%; padding: 5px;">           Date of Mailing of this International Search Report  <div style="border: 1px solid black; padding: 2px; display: inline-block;">DEC 1991</div> </td> </tr> <tr> <td style="padding: 5px;">           International Searching Authority  <div style="border: 1px solid black; padding: 2px; display: inline-block;">ISA/US</div> </td> <td style="padding: 5px;">           Signature of Authorized Officer  <div style="text-align: center;">              Ardin Marschel           </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="border: 1px solid black; padding: 2px; display: inline-block;">25 November 1991</div>	Date of Mailing of this International Search Report <div style="border: 1px solid black; padding: 2px; display: inline-block;">DEC 1991</div>	International Searching Authority <div style="border: 1px solid black; padding: 2px; display: inline-block;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;">              Ardin Marschel           </div>					
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## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	The Journal of Biological Chemistry, Volume 265, Number 19, issued 05 July 1990, Stromberg et al., "Characterization of the Binding of Propionibacterium Granulosum to Glycosphingolipids Adsorbed on Surfaces", pages 11244-11250, see especially the abstract and Table I on page 11246.	1,4,5, 7-9,11-14 2-3,10, 15-17
X Y	Proceedings of the National Academy of Sciences, Volume 85, issued July 1988, Stromberg et al., "Identification of carbohydrate structures that are possible receptors for <u>Neisseria gonorrhoea</u> ", pages 4902-4906, see especially the abstract and Table 1 on page 4903.	1,2,4,5, 8,9,11-14 10,15-17
Y	Proceedings of the National Academy of Sciences, Volume 87, issued January 1990, Paruchuri et al., "Identification and characterization of a <u>Neisseria gonorrhoea</u> gene encoding a glycolipid-binding adhesion" pages 333-337, see especially the abstract and "Materials and Methods" section.	1-17, 24-29, 32-52, 54-56
Y	US, A, 4,795,803 (LINDBERG ET AL.) 03 January 1989, see especially the abstract.	1-17, 24-29, 32-52, 54-56
A	US, A, 4,661,345 (TUOMANEN) 28 April 1987, see the entire document.	1-17, 24-29, 32-52, 54-56
A	US, A, 4,762,824 (KALLENIOUS ET AL.) 09 August 1988, see the entire document.	1-17, 24-29, 32-52, 54-56
Y	US, A, 4,464,360 (LEFFLER ET AL.) 07 August 1984, see entire document.	24-29

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Biochimica et Biophysica Acta, Volume 832, issued 1985, Jacobs et al., "Effect of Chemical Modifications on the K99 and K88ab fibrillar adhesions <u>Escherichia coli</u> ", pages 148-155, see especially the sentence bridging pages 148 and 149.	1-17, 24-29, 32-52, 54-56
Y	Journal of Clinical Microbiology, Volume 25, Number 2, issued February 1987, S. Fnerback et al., "Binding to Galactoseal-4 Galactoseß-Containing Receptors as Potential Diagnostic Tool in Urinary Tract Infection", pages 407-411, see especially the abstract.	1-17, 24-29, 32-52,
A	Journal of Cellular Physiology, Volume 141, issued 1989, J. Inokuchi et al., "Effects of D-throe-PDMP, an Inhibitor of Glucosylceramide Synthetase, an Expression of Cell Surface Glycolipid Antigen and Binding to Adhesive Proteins by B16 Melanoma Cells", pages 573-583, see especially the abstracts.	1-17, 24-29, 32-52, 54-56
Y	Journal of Clinical Microbiology, Volume 25, Number 2, issued February 1987, DeMan et al., "Receptor Specific Agglutination Tests for Detection of Bacteria that Bind Globoseries Glycolipids", pages 401-406, see especially the abstract.	1-17, 24-29, 32-42
Y	Infection and Immunity, Volume 56, Number 10, issued October 1988, A.E. Wold et al., "Attachment of <u>Escherichia coli</u> via Mannose- or Gal 1 4 Galß-Containing Receptor to Human Colonic Epithelial Cells", pages 2531-2537, see especially the abstract.	1-17, 24-29, 32-42,
Y	Infection and Immunity. Volume 51, Number 3, issued March 1986, Lomborg et al., "Influence of Blood Group on the Availability of Receptors for Attachment of Receptors for Attachment of Uropathogenic <u>Escherichia coli</u> ", pages 919-926, see especially the abstract.	1-17, 24-29, 32-42



## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	NATURE, Volume 328, issued 02 July 1987, Lindberg et al., "Localization of the receptor-binding protein adhesion at the tip of the bacterial pilus", pages 84-87, see especially the abstract and the <u>pap</u> gene cluster in Figure 1 on page 85.	1-17, 24-29, 32-52, 54-56
X Y	Journal of Biological Chemistry, Volume 254, Number 18, issued 25 September 1979, K.-A. Karlsson et al., "Structural Characterization of Lactotetracyclamide, a Novel Glycosphingolipid Isolated from Human Meconium", pages 9311-9316, see especially the abstract.	1-5 6-17, 24-29
X Y	Archives of Biochemistry and Biophysics, Volume 270, Number 1, issued April 1989, Kyogashima et al., " <u>Escherichia coli</u> K99 Binds to N-Glycolylsialoparagloboside and N-Glycolyl-GM3 Found in Piglet Small Intestine", pages 391-397, see especially the abstract and the first paragraph on page 391 and Table I on page 392.	1-5, 8, 9, 11-14, 10, 15-17, 24-29
A	Journal of Biological Chemistry, Volume 264, Number 16, issued 05 June 1989, Roberts et al., "Sialic Acid-dependent Adhesion of <u>Mycoplasma pneumoniae</u> to Purified Glycoprotein", pages 9289-9293, see especially the entire disclosure.	1-17, 24-29, 32-52, 54-56
X Y	Proceedings of the National Academy of Sciences, Volume 85, issued August 1988, Krivan et al., "Many Pulmonary Pathogenic Bacteria bind Specifically to the Carbohydrate Sequence GalNAc $\beta$ 1-4 Gal found in some Glycolipids", pages 6157-6161, see especially the abstract and Table 1 on page 6158.	1-5, 8, 9, 11-14, 17 24, 26-29
X	EP, A, 0,352,766 (VALLE ET AL.) 31 January 1990, see especially Table 1 on page 2 and claims 1-20.	1

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(X) (a) for the following reasons:

1. ☐ Claim numbers . . . . . because they relate to subject matter<sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . . . . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>13</sup>, specifically:

3. ☐ Claim numbers . . . . . because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

See attachment entitled "ATTACHMENT A".

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:  
1-17, 24-29, 32-52, and 54-56.

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

☒ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ATTACHMENT A (CONTINUATION OF PART VI)

I. Claims 1-17, drawn to a receptor, detection method, and kit; classified in Class 435, subclass 34.

II. Claims 18 and 19, drawn to a method of removing pathogens from solution, classified in Class 435, subclass 261.

III. Claims 20-23, drawn to an apparatus for removing pathogens from a liquid, classified in Class 435, subclass 299.

IV. Claims 24-29, drawn to a pharmaceutical composition and related treatments, classified in Class 514, subclass 62.

V. Claims 30 and 31, drawn to a method of inhibiting adherence, classified in Class 435, subclass 178.

VI. Claim 32, drawn to a method of adhesin preparation using receptor, classified in Class 530, subclass 413.

VII. Claims 33-39, 41, 52, and 54, drawn to an adhesin protein, classified in Class 530, subclass 350.

VIII. Claims 40, 42, 55, and 56, drawn to a vaccine containing adhesin protein, classified in Class 514, subclass 2.

IX. Claims 43 and 44, drawn to a method of production of DNA encoding an adhesin protein, classified in Class 435, subclass 91.

X. Claims 45-51, drawn to an adhesin protein DNA, classified in Class 536, subclass 27.

XI. Claim 53, drawn to a method of production of adhesin protein, classified in Class 435, subclass 69.1.

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